[CANCER RESEARCH 57, 28–31, January 1, 1997]

Advances in Brief

Coexpression of Grb7 with Epidermal Growth Factor Receptor or Her2/erbB2 in Human Advanced Esophageal Carcinoma[1]

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Abstract

Growth factor receptors transmit intracellular signals that may be important in carcinogenesis. The Grb7 protein was recently identified as a substrate of the epidermal growth factor receptor and related Her2/erbB2 receptor-linked tyrosine kinase activity. The *Grb7* gene has been found to be coamplified with Her2/erbB2 in breast carcinomas. In this study, Grb7 expression was studied in 32 human esophageal cancers. A human Grb7 cDNA encoding for N-terminal amino acids was isolated and found to be 90% homologous to the murine counterpart. Although there was no amplification of the *Grb7* gene in esophageal cancers, Grb7 mRNA was found to be overexpressed in 14 cancers (43.8%) but not in adjacent normal esophageal mucosa. It is noteworthy that coexpression of Grb7 with epidermal growth factor receptor or Her2/erbB2 was detected in 10 esophageal carcinomas (31.3%) and was significantly related to extramucosal tumor invasion ($P = 0.02$), whereas such a relationship was not shown by each sole expression. These findings suggest a possible relationship of Grb7 signaling in association with expression of tyrosine kinase receptors in aggressive human esophageal cancer.

Introduction

A key mechanism for induction of cellular growth and transformation involves the transmission of intracellular signals following activation of receptor-linked tyrosine kinases by growth factors (1). Subsequent tyrosyl phosphorylation of intracellular substrates leads to an interaction with SH2[3] domains of downstream signaling molecules (2). Binding of signal transduction molecules to such phosphotyrosine motifs will affect the properties of SH2-containing proteins as exemplified by direct stimulation of enzymatic activity and relocalization of proteins within the cytoplasm and tyrosyl phosphorylation of other downstream signal transduction molecules (2). In this regard, Schlessinger and colleagues (3–5) have utilized tyrosyl phosphorylated EGFRs as a probe for cloning SH2-containing proteins that will bind to the activated receptor. Subsequently, several Grb proteins have been identified and include proteins such as Grb1, the p85 subunit of phosphatidylinositol-3 kinase, and Grb2 (also called Ash), a molecule that regulates the SOS guanine exchanging factor known to be important in *Ras* activation. In addition, Grb3, Grb4, Grb5, Grb6, and Grb9 have been isolated and found to have the same characteristics of the signaling proteins Crk, Nck, Fyn, phospholipase C-γ1, and Syp, respectively (5, 6). With respect to their possible role in oncogenesis, we have previously found that cellular transformation induced by IRS-1 requires an interaction with both Grb2 and Grb9, respectively. Thus, these binding motifs of the IRS-1 protein have a functional role in producing cellular transformation (7).

The Grb7 is a newly identified SH2-containing protein that binds avidly to the activated EGFR (5). Murine Grb7 protein is composed of a SH2 domain at the carboxyl (C) terminus and PH domain that has also been found in various signaling molecules such as IRS-1. In addition, a protein phosphatase 2B (P2B2)-like proline-rich sequence is also present in the amino (N)-terminal region of the Grb7 protein. The proline-rich sequence may be a target region for binding by other signaling molecules containing SH3 domains. The Her2/erbB2 protein is another receptor-type tyrosine kinase with similar properties to the *neu* oncogene, and this molecule has a high degree of structural homology to the EGFR, since it contains a cytoplasmic binding region that reacts with signaling molecules containing SH2 domains. Previously, Stein *et al.* (8) revealed that the Grb7 protein will bind tightly to the Her2/erbB2 protein, and the *Grb7* gene was frequently coamplified with the *Her2/erbB2* gene. With respect to tissue expression, Grb7 expression is normally present only in liver, kidney, and gonads. However, most breast carcinoma cell lines and tissues express Grb7 at the mRNA and protein level. Thus, there needs to be further studies to determine whether Grb7 expression is important in carcinogenesis.

Human esophageal carcinoma is often an aggressive tumor with a poor prognosis (9). It has been previously suggested that several growth factors including EGF, TGF-α, and platelet-derived growth factor might play a role in esophageal carcinogenesis by either an autocrine or paracrine process; however, the molecular mechanisms and signaling molecules involved has yet to be clarified (10). Previous studies have indicated that overexpression of the EGFR is a frequent finding in this disease and the receptor tyrosine kinase activated by EGF and TGF-α, and EGFR overexpression has been identified as a prognostic factor in esophageal carcinomas (11). Additionally, expression of Her2/erbB2 has been detected in various esophageal tumors also (12, 13). It is likely that the activated EGFR and Her2/erbB2 will transmit the growth signals and contribute to the transformation process. In the present investigation, we determined whether Grb7 was expressed in esophageal tumors on the basis of either gene amplification or enhanced transcriptional activity. More important, the significant Grb7 and EGFR or Her2/erbB2 coexpression was explored in tumor tissues compared to the adjacent normal esophageal mucosa. Our results suggest that there is an important correlation between tumor invasion and possible signaling by receptor tyrosine kinases through the Grb7 in human esophageal carcinoma.

Materials and Methods

To clone a cDNA fragment of human Grb7, sequences homologous to P2B2 and PH domain of the murine Grb7 were used as PCR primers shown in Fig.

Received 9/16/96; accepted 11/14/96.

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[1] This work was supported by a grant from the Fukuoka Cancer Society. The represented sequence has been registered with GenBank, accession no. D87513.

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[3] The abbreviations used are: SH, src homology; Grb, growth factor receptor bound; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; TGF, transforming growth factor; P2B2, protein phosphatase 2B; GAPDH, glyceraldehyde-3-phosphatase dehydrogenase; PH, pleckstrin homology; IRS-1, insulin receptor substrate 1; RT, reverse transcription.

1 (5). Total RNA was extracted from ZR-75-30 human breast carcinoma cells known to overexpress Grb7 mRNA and protein (8), followed by RT-PCR amplification as described previously (14). The PCR products were cloned using a TA cloning kit (Invitrogen), and DNA sequences were determined using the Sequenase version 2.0 (United States Biochemical Corp.). To analyze the status of Grb7 expression and its relationship to associated molecules in clinical samples, we studied 32 primary squamous cell esophageal tumors from the Saitama Cancer Center (Saitama, Japan) obtained at the time of surgical resection. Genomic DNA and total cellular RNA were extracted according to standard methods. Amplification of the *Grb7* gene was assessed by PCR as described previously (8). Cellular expression of Grb7, EGFR, and Her2/erbB2 was analyzed after performing the RT reaction using the following primers for 25-cycle PCR as described previously (15, 16): Grb7, 5′-CCTCTCCTCATC-CCAACCAC-3′ and 5′-GCAGCCTGCACTTCCACCAC-3′ (product size, 380 bp); EGFR, 5′-CGTTGCTGGCTGCGCTCTG-3′ and 5′-AGCCACCTC-CTGGATGGTC-3′ (product size, 221 bp); and Her2/erbB2, 5′-GCTGGCTC-CGATGTATTTGATG-GT-3′ and 5′-GTTCTCTGCCGTAGGTGTCCCT-TT-3′ (product size, 495 bp). As a control mRNA, we performed RT-PCR of GAPDH as reported previously (14): 5′-GTCAACGGATTTGGT-CTG-TATT-3′ and 5′-AGTCTTCTGGGTGGCAGTGAT-3′ (product size, 560 bp). All PCR primers were selected to span the introns to detect specific mRNA sequences.

Results and Discussion

The murine Grb7 contains a P2B2-like proline-rich sequence at the NH_2 terminus and a SH2 domain in the C-terminal region of the molecule (8). The region located between these two motifs has an homology to the central region of *Caenorhabditis elegans* F10E9.6. In both regions, a consensus sequence for a PH domain has been identified (17). The strategy used for cloning of a human Grb7 partial cDNA by PCR, therefore, incorporates sequences homologous to the P2B2 and PH domains as primers (Fig. 1). The RT-PCR was performed on RNA extracted from ZR-75-30 human breast carcinoma cells previously found to overexpress the Grb7 protein (8). Using these approaches, a 960-bp fragment was isolated and characterized. The nucleotide sequence of the human Grb7 partial cDNA is presented in Fig. 1. (The sequence represented was deposited with GenBank accession no. *D87513*.) The predicted amino acid sequence is highly homologous (89.6%) to the murine Grb7 protein. In addition, the proline-rich sequence is also retained in the human form of the Grb7 molecule. Finally, the PH domain found in human Grb7 is 95.7% similar to the PH domain of murine Grb7. Although the function(s) of PH domains has not been clarified, recent experiments suggest that the PH region interacts with $G\alpha\beta$ subunits (18) and phospholipids (19) and may be important in the association with signal transduction complexes.

It is of interest that the murine *Grb7* gene localizes to chromosome 11 near the *Her2/erbB2* locus. In addition, the *Grb7* gene is frequently coamplified with the *Her2/erbB2* gene in human breast carcinoma cells (8). Genomic DNA extracted from human esophageal tissues was analyzed using the Grb7 cDNA probe to determine whether there was gene amplification in these tumors (8). However, we were unable to detect *Grb7* gene amplification in 32 tissue samples of human esophageal carcinoma. These results are consistent with the observation that *Her2/erbB2* gene amplification has not been previously found in squamous cell carcinoma of the esophagus (12). Stein *et al.* (8) reported that overexpression of Grb7 mRNA in human breast carcinoma cells was not due to gene amplification. Based on their results, we analyzed the pattern of Grb7 expression in esophageal carcinomas by RT-PCR using the specific primers outlined in Fig. 1. Grb7 mRNA was not detectable in normal esophageal mucosa, although there is specific expression in liver, kidney, and gonadal tissues. In contrast, as demonstrated in Fig. 2, Grb7 mRNA expression was found in 14 esophageal

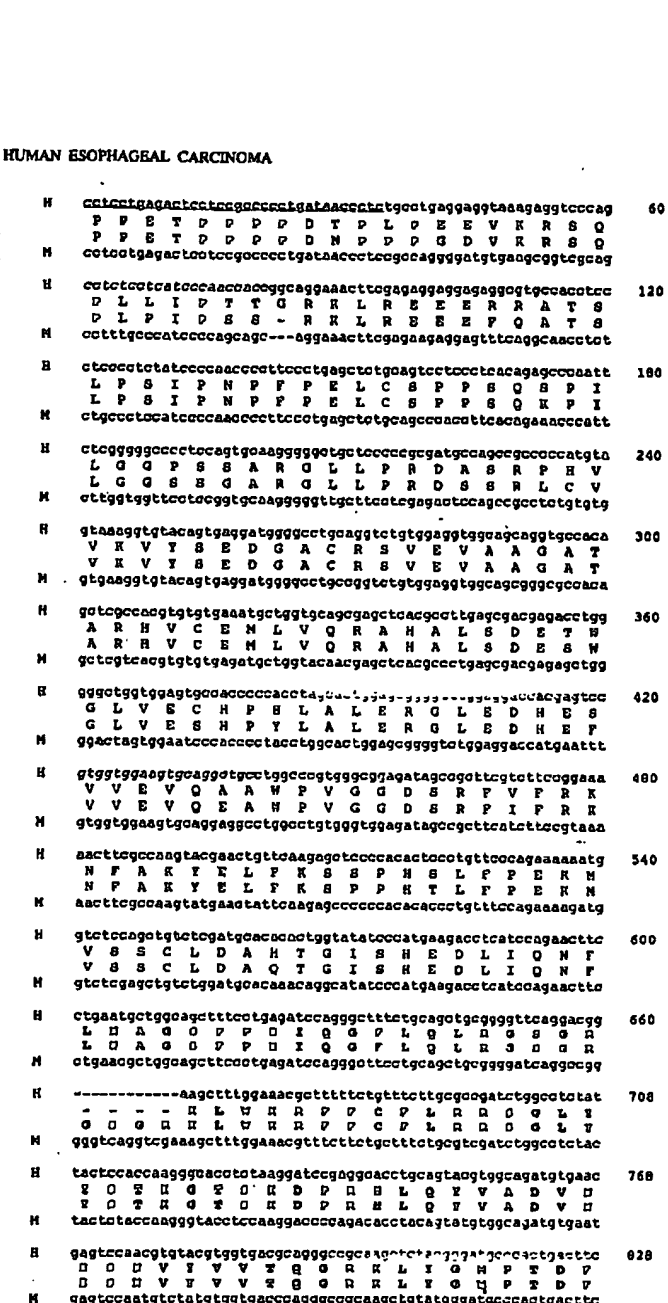

Fig. 1. Partial nucleotide sequence and predicted amino acid sequence of human Grb7 (*H*) compared to murine Grb7 (*M*) as reported by Stein *et al.* (8). A human Grb7 cDNA was isolated from ZR-75-30 human breast carcinoma cells (12). The *underlined* nucleotide sequence was used to construct PCR primers to amplify a 960 bp of human Grb7 cDNA. The nucleotide sequence presented in *italics* was used to detect Grb7 expression in human esophageal tissues by RT-PCR (380 bp). The predicted amino acid sequence of human Grb7 protein is 93.8% homologous to the murine Grb7 sequence. The ***bold*** sequences indicate the proline-rich P2B2-like sequence and the PH domain. The represented sequence was deposited with GenBank accession no. *D87513*.

tumors (43.8%). To address the possible clinical significance of this finding, we compared the relationship of Grb7 mRNA expression to the clinicopathological characteristics of sex, age, tumor size, histological differentiation, depth of invasion, nodal status, metastasis, histological stage, and prognosis. However, we found

no relationship of Grb7 mRNA expression to any of these clinical features.

The Grb7 protein has been shown to be a substrate for either EGFR or Her2/erbB2, since both tyrosine kinases have highly similar cytoplasmic domains that will bind to SH2 containing signal transduction molecules (8). In this regard, overexpression of EGFR or the Her2/erbB2 was detected in 18 esophageal tumors. Indeed, 12 tumors were found to express the EGFR whereas 8 others expressed the Her2/erbB2 oncoprotein. Overexpression of both EGFR and Her2/erbB2 was found in two tumor samples. It is noteworthy that previous reports have also provided evidence for overexpression of these receptors in human esophageal carcinomas (10, 12), and one study emphasized that overexpression of EGFR was a prognostic factor in this disease (11). In our study, there was no association between overexpression of EGFR or Her2/erbB2 with the clinical and pathological course of esophageal carcinoma. However, coexpression of Grb7 with EGFR or Her2/erbB2 was found in 10 tumors (Fig. 2) and was significantly

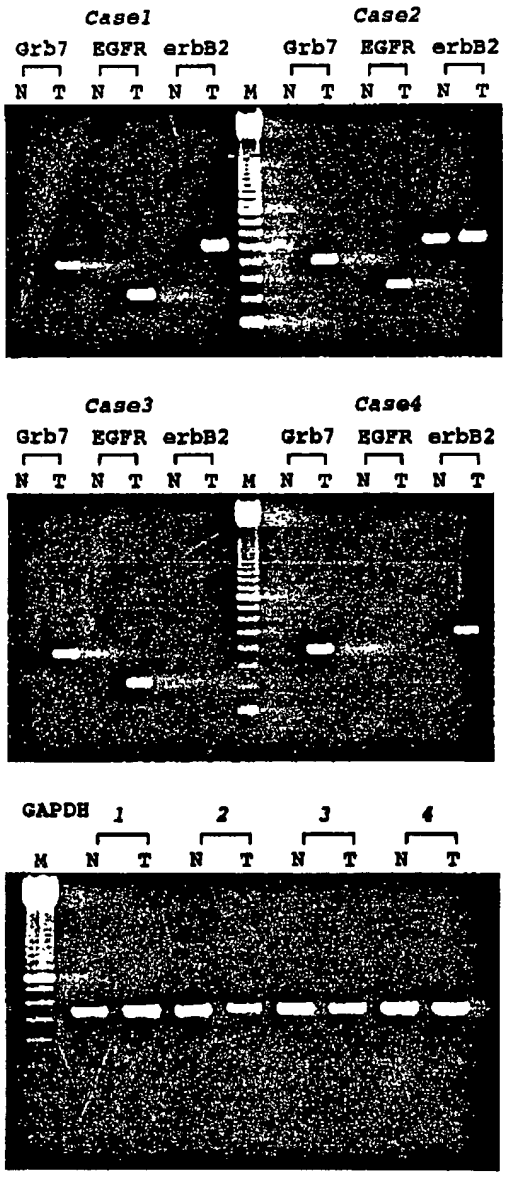

Fig. 2. Expression of Grb7, EGFR, Her2/erbB2, and GAPDH in human esophageal carcinomas (*T*) and normal adjacent mucosa (*N*) as measured by RT-PCR. Case numbers are *italic*.

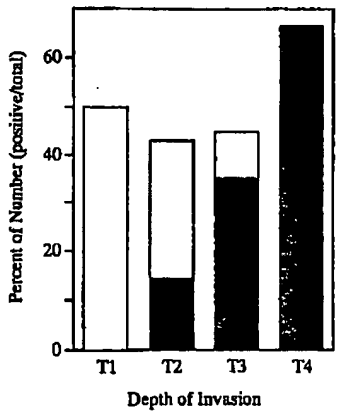

Fig. 3. Relationship between Grb7 expression and tumor invasion of human esophageal carcinoma. *Columns*, percentage of tumors positive for Grb7 expression. *Filled columns*, percentage of tumors positive for coexpression of Grb7 with EGFR and/or Her2/erbB2. The tumor invasion depth was classified using the UICC standard where T_1 is mucosa or submucosa, T_2 is muscularis propria, T_3 is adventitia, and T_4 is extra-adventitial spread.

related to the depth of tumor invasion (Fig. 3). Indeed, the coexpression of Grb7 with these receptors was detected in 66.7% of advanced esophageal carcinomas with extramucosal invasion but in none of the tumors confined to the esophageal mucosa ($P = 0.02$, Mann-Whitney U test). Thus, coexpression of these signal transduction molecules was associated with tumor invasion. In support of this conclusion is our findings in 14 of 18 tumor cell lines derived from human esophageal carcinomas where coexpression of Grb7 with these two receptor tyrosine kinases was positively associated with the degree of tumorigenicity in nude mice.[4] Taken together, these findings suggest that intracellular signals transmitted by such receptor tyrosine kinases through Grb7 may be directly involved in tumor progression.

Human esophageal carcinoma is known to be highly invasive (9), and our studies suggest that coexpression of Grb7 with these two receptors may have clinical significance in this regard. However, the function of Grb7 in this process has yet to be defined. It will be important to clarify the molecular effects induced by Grb7 expression particularly with respect to whether a growth or metastatic signal is relayed to Grb7 by the activated EGFR and/or erbB2. In this regard, the proline-rich sequences of the NH_2 terminus region of Grb7 protein may also interact with SH3 domains of other signal transduction molecules (2). Additional studies will be needed to be directed toward identifying such downstream signal transduction molecules that may interact with Grb7 and transmit oncogenic signals (20). Such investigations will further clarify the role of Grb7 as a contributor to invasive characteristics of human esophageal carcinomas.

Acknowledgments

We thank K. Ogata and K. Mimori for technical assistance.

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